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Phylogeny of cyclic nitramine-degrading psychrophilic bacteria in marine sediment and their potential role in the natural attenuation of explosives

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Abstract

Previously we reported on in situ mineralization of cyclic nitramine explosives including hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in marine sediment from Halifax Harbour. In the present study, we isolated several novel psychrophilic bacteria from the sediment with optimal growth temperature at 10 or 15 °C. Phylogenetic analysis of their 16S rRNA genes identified the isolates as members of the gamma and delta subdivisions of Proteobacteria, Fusobacteria and Clostridiales. The isolates mineralized 3.7–45.2% of RDX (92 μM) in 82 days of incubation at 10 °C under oxygen-limited or anaerobic conditions with the gamma subdivision isolates demonstrating the highest mineralization (45.2% of total C). Removal of RDX by all isolates was accompanied by the formation of all three nitroso derivatives, with the mono nitroso derivative (MNX) being the major one. Isolates of the delta proteobacteria and Fusobacteria removed HMX with concurrent formation of the mononitroso derivative (NO-HMX). Using resting cells of isolates of the gamma subdivision, methylenedinitramine (MEDINA) and 4-nitro-2,4-diazabutanal (NDAB) were detected, suggesting ring-cleavage following denitration of either RDX and/or its initially reduced product, MNX. These results clearly demonstrate that psychrophilic bacteria capable of degrading cyclic nitramines are present in the marine sediment, and might contribute to the in situ biodegradation and natural attenuation of the chemicals.

Keywords: Cyclic nitramine explosive; RDX biodegradation; Psychrophilic bacteria; Marine bacteria; Phylogeny; *Shewanella*; Sulfate-reducing bacteria; Methylenedinitramine; 4-Nitro-2,4-diazabutanal; Marine sediment

1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are two widely-used cyclic nitramine explosives (Fig. 1). They are toxic to various terrestrial and aquatic organisms [1–3]. Sinking of military ships and dumping of munitions during the two World Wars have resulted in the undersea deposition of unexploded ordnance (UXO), where leaching constitutes a major source of contamination of marine and estuarine sediments [4]. Trace amounts of the explosive TNT were previously detected in Halifax Harbor [4].

Marine sediment is mainly a cold, oxygen-limited (upper layer) or anaerobic environment (lower layer) where psychrophilic or psychrotrophic anaerobic bacteria can be found [5–7]. Thus far no psychrophilic marine bacteria have been described for their ability to

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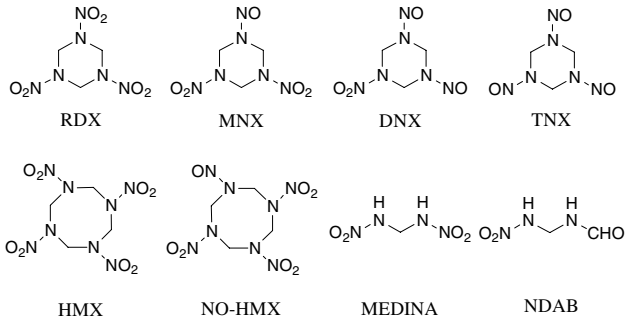

Fig. 1. Chemical structures of cyclic nitramines and their biotransformation products.

transform or degrade cyclic nitramines. A few terrestrial mesophilic microorganisms such as *Rhodococcus* sp. [8–10], *Klebsiella pneumoniae* strain SCZ-1 [11] and the fungus *Phanerochaete chrysosporium* [12–15] degrade RDX. Under anaerobic conditions, bacteria such as *Clostridium* [16–19], *Desulfovibrio* [18] and some members of the *Enterobacteriaceae* family [20,21], removed RDX with little mineralization [17,20,21]. High RDX mineralization (72% of total C) was found in one mesophilic anaerobic isolate, *K. pneumoniae* strain SCZ-1, obtained from anaerobic sludge [11].

Recently, we showed that RDX and HMX could be mineralized in anoxic and cold marine sediment from a former ammunition-dumping site at Emerald Basin near Halifax Harbor in the Atlantic Ocean [22]. The goal of the present study was to identify the bacteria in the sediment that can degrade RDX and HMX anaerobically at low temperature. Generated data will provide insight into the natural attenuation of these chemicals and thus help optimize in situ remediation strategies for contaminated marine environments.

2. Materials and methods

2.1. Reagents and media

RDX (99% pure), HMX (98% pure), [UL-^{14}C]-RDX (chemical purity, >98%; radiochemical purity, 96%; specific radioactivity, 28.7 μCi mmol^{-1}) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX, 99% pure) were provided by Defense Research and Development Canada (DRDC), Quebec, Canada. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX, 98% pure) and 4-nitro-2,4-diazabutanal (NDAB) (99% pure) were provided by R.J. Spanggord from SRI International (Menlo Park, California, USA), and methylenedinitramine (MEDINA) was purchased from the rare chemical department, Aldrich, Canada. All other chemicals were reagent grade.

The marine salts medium was prepared as described previously [22]. Solid marine medium was prepared by dissolving Brewer Anaerobic Agar (Becton Dickson, Sparks, MD, USA) in marine salts medium. Liquid marine media used in the present study were marine broth 2216 (Becton Dickinson, Sparks, MD) [7], or prepared by dissolving 0.1 or 1 g each of yeast extract, bacto peptone, and glucose in 1 l marine salts medium supplemented with or without 2 g (dry weight) autoclaved sediment.

2.2. General conditions for biodegradation and biotransformation

Growth of all isolates and biotransformation tests were conducted statically at 10 °C unless otherwise indicated. Aqueous solubility at 10 °C was 22 mg l^{-1} (100 μM) for RDX and 1.7 mg l^{-1} (5.6 μM) for HMX. Initial substrate amounts in media were 20 mg l^{-1} (90 μM) for RDX and 2 mg l^{-1} (6.7 μM) for HMX. Media were maintained below 10 °C prior to inoculation for growth or transformation.

2.3. Isolation and characterization of microorganisms from sediment

Sampling and characterization of marine sediment (redox potential, 90 mV; pH, 7.7; total organic carbon, 12 g kg^{-1} (dry weight) of sediment; NH_3, 7.7 mg kg^{-1} (dry weight) of sediment) from the Emerald Basin (215 m deep, 50 nautical miles from Halifax harbor, Nova Scotia, Canada) were reported previously [22]. Previously, sediment taken from the same site at a nearby location showed the presence of a trace of TNT, but neither RDX nor HMX were detected [4]. The sealed sediment samples are kept in a cold room at 4 °C. Only the interior portion of the sediment sample was used for bacterial isolation to avoid artifacts that may occur near the surface of the sample. In an anaerobic glove box, sediment (wet weight, 25 mg; dry weight, 9.5 mg) suspended in marine salts medium (50 μl) was spread on the above solid marine media (Section 2.1), followed by aerobic or anaerobic incubation at 10 °C for up to 28 days. The capacity of detected colonies to degrade RDX or HMX was evaluated based on removal of the two chemicals after two weeks of aerobic or anaerobic incubation in 2 ml of marine broth 2216 or other liquid marine media (Section 2.1). We also tried the in situ diffusion growth chamber method [23] to isolate bacteria, but found it not suitable for growth of psychrophiles.

Biochemical tests of isolates were performed using the Rapid 20E and Rapid ID 32A test strips (bioMèrieux, Montreal, Canada) and the standard protocols described by Smibert and Krieg [26]. Optimal growth temperatures of the present isolates were determined based on their growth rates in marine broth 2216 at 4, 10, 15, 22, 30 and 37 °C, using a protocol described by Bowman [7].

2.4. Bacterial growth and cyclic nitramines biodegradation

All marine isolates (HAW-EB1–5, HAW-EB17, 18 and 21) grew well in marine broth 2216 or other peptone and yeast extract based media that contained marine salts such as NaCl (2% w/v) [22]. Poor growth was observed in media that contained less NaCl (0.5%) and were not supplemented with typical marine salts. Therefore marine broth 2216 was used for growth of all positive isolates in subsequent studies unless otherwise indicated. The procedure for biodegradation tests by growing cells of isolates in group I under oxygen-limited conditions was as follows: 2 ml of a 5-day aerobically-grown liquid culture was inoculated into 18 ml of cyclic nitramine-containing medium in 60 ml sealed serum bottles with air in the headspace, followed by static incubation. After 5 days, the cultures grew to an OD of 0.65 (HAW-EB2), 0.84 (HAW-EB3) or 0.65 (HAW-EB4) from an initial OD of 0.05–0.07. The biomass by weight was 2.5–4.1 $g\,l^{-1}$ (of wet cells) or 0.5–0.8 $g\,l^{-1}$ (of dry cells). For obligatory anaerobic isolates, HAW-EB17, 18 and 21, media were made anaerobic prior to inoculation by degassing and charging with argon three times using a protocol that we described previously [17]. To avoid abiotic reduction, no anaerobic reducing agents were added. After 43 h of incubation, isolate HAW-EB 21 grew to an OD of 1.2 from an initial OD of 0.1. Whereas isolate HAW-EB18 grew to 9.0×10^8 CFU (colony forming unit) ml^{-1} from an initial 1.0×10^8 CFU ml^{-1} after 5 days of incubation. Microcosms were supplemented with [UL-^{14}C]-RDX (0.038 μCi) to measure mineralization (liberated $^{14}CO_2$) using a Tri-Carb 4530 liquid scintillation counter (LSC, model 2100 TR, Packard Instrument Company, Meriden, CT, US) [25].

For biotransformation tests with cell suspensions, isolates HAW-EB2, HAW-EB4 and HAW-EB5 were incubated aerobically in marine broth 2216 in foam-plugged flasks at 200 rpm on a rotary shaker for 5 days (final OD: 1.0–1.2). Cells were harvested by centrifugation at 12,000*g* for 30 min and washed once with marine salts medium. The wet cell weight of isolates HAW-EB2, HAW-EB4 and HAW-EB5 from 1.26 l of the above liquid cultures was 5.2, 4.8 and 3.2 g, respectively. Cells were suspended in 50 ml of marine salts medium, and kept at 4 °C until used. Biotransformation media were composed of 5 ml of cell suspension, 25 ml of marine salts medium, and 30 μl of stock solution of RDX (20,000 $mg\,l^{-1}$) in 60 ml serum bottles. The cell concentrations for the isolates were (isolate, $g\,l^{-1}$ (of wet cells), $g\,l^{-1}$ (of dry cells)): HAW-EB2, 17, 3.4; HAW-EB4, 16, 3.2; HAW-EB5, 11, 2.1. Bottles were degassed briefly and recharged three times with argon. Separate microcosms were set up for analysis of the gaseous product nitrous oxide (N_2O). Removal of N_2O (initial amount, 0.3–0.5 μmole in 55 ml headspace) and nitrite (initial concentration, 3.1 mM) were conducted under the same conditions except that the reaction volume was 6 ml. Autoclaved cell suspensions were used as controls.

2.5. Phylogenetic analyses of 16S rRNA gene sequences

Colonies grown on marine agar were picked for extraction of total DNA and PCR amplification of 16S rRNA genes according to standard molecular biology methods [27]. Sequences, with a length ranging from 1170 to 1290 bases, of 16S rRNA genes were compared to published sequences using the BLAST. The 16S rRNA gene sequences of the isolates and those of closely related standard strains were aligned using ClustalX(1.81). The neighbor-joining method (in the MEGA2 package [28]) based on the pair wise nucleotide distance of Kimura 2-parameter, was used to build the phylogenetic trees. The number of bootstrap repetitions was 2000.

2.6. Analyses of cyclic nitramines and their products

The concentrations of RDX, MNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX, Fig. 1), TNX and HMX were analyzed by HPLC as described previously [11,25,29]. NDAB and MEDINA were determined on an AnionSep Ice-Ion-310 Fast organic acids HPLC column (6.5 × 150 mm, Cobert associates chromatography products, St-Louis, MI) at 225 nm and 35 °C. The mobile phase was 1.73 mM sulfuric acid at a flow rate of 0.6 ml/min. The elution times of MEDINA and NDAB under the above conditions were 6.4–6.8 and 7.2–8.0 min, respectively. The methods for analyses of NO_2^-, N_2O, NH_4^+ and HCHO were described in earlier reports [11,25]. All tests were performed in triplicate.

3. Results and discussions

3.1. Characterization of cyclic nitramine-degrading bacteria in marine sediment

Two groups (group I and II) of bacteria capable of degrading RDX and HMX at low temperature (4–10 °C) were isolated from the sediment that previously showed potential for degradation of the two chemicals [22].

All RDX-degrading isolates within group I (RDX^+HMX^-) were isolated from the initially detected aerobically grown colonies. They accounted for a very small fraction (1.5 ± 1.1%) of the total viable aerobic count (10 °C, $1.7[\pm 0.2] \times 10^4$ CFU g^{-1} (dry weight) of sediment) of the present sediment. The total viable aerobic count of the present sediment fell within the range of those (10^2–10^6 CFU g^{-1} (dry weight) of sediment) found in other marine sediments [5]. Most

($83 \pm 3\%$) of the initially detected aerobic colonies were extremely slow growing and unable to be sub-cultured on solid marine medium.

Isolates of group I were motile, Gram-negative, oxidase-, catalase- and nitroreductase-positive, and short-rod shaped bacteria. They were different in color (orange or dark yellow) and/or cellular sizes (isolate, diameter × length [μm], colour): HAW-EB1, $0.25 \times (1.5–2.5)$, orange; HAW-EB2, $0.25 \times (0.5–1.5)$, dark orange; HAW-EB3, $0.25 \times (0.5–1.5)$, orange; HAW-EB4, $0.5 \times (0.5–1.5)$, dark yellow; HAW-EB5, $0.25 \times (1.5–3.0)$, dark orange, and in their enzyme content and/or growth substrate profiles (data not shown). All strains grew well aerobically at temperatures of 4, 10, 15 and 21 °C, but did not grow at 30 °C and above. For all group I isolates, the optimal aerobic growth rate ($4.5–6.6 \times 10^6$ cells $ml^{-1}\,h^{-1}$) was at 10 °C, indicating that they were psychrophiles.

All five isolates favored aerobic conditions to grow, and their growth under strictly anaerobic conditions was poor. These isolates degraded RDX under oxygen-limited static incubation conditions (1 atm air in 40 ml headspace of 60-ml sealed bottles) (Fig. 4, HAW-EB2-4), not in fully shaken aerobic liquid culture (in foam-plugged flask). We found that the headspace above RDX-degrading static microcosms in sealed bottles contained about 5–17% oxygen (of total gas) after 7 days of incubation (Fig. 4). Aerobically-grown cells of these isolates also degraded RDX under anaerobic conditions (Fig. 6).

RDX and HMX-degrading isolates within group II (RDX^+HMX^+), four in total, grew under strictly anaerobic conditions. They were isolated from (13% of the total) the initially detected anaerobic colonies ($3.9[\pm 0.41]$ CFU $\times 10^3\,g^{-1}$ (dry weight) of sediment), most (73%) of which were able to be sub-cultured on solid marine medium. Of the total recovered anaerobic isolates, all the obligate anaerobes tested degraded cyclic nitramines. Isolate HAW-EB18 was a motile, rod-shaped (0.5–1.5 μm long with a diameter of 0.25 μm), catalase-positive and whitish (or blackish later) sulfate-reducing bacterium. Isolate HAW-EB21 was a non-motile, oxidase and catalase negative, rod-shaped (0.5–1.0 μm long with a diameter of 0.5 μm) and transparent fermentative bacterium, producing H_2 and H_2S when grown on peptone and yeast extract in marine medium 2216. Compared to isolates of group I, the two strictly anaerobic isolates HAW-EB18 and HAW-EB21 could grow at wider range (4, 10, 15, 21 and 30 °C) of temperatures with better and similar growth found at three temperatures: 10, 15 and 21 °C. Both isolates were also considered to be psychrophiles.

Another strictly anaerobic isolate, HAW-EB17, was a Gram-negative, long rod shaped (4–20 μm long with a

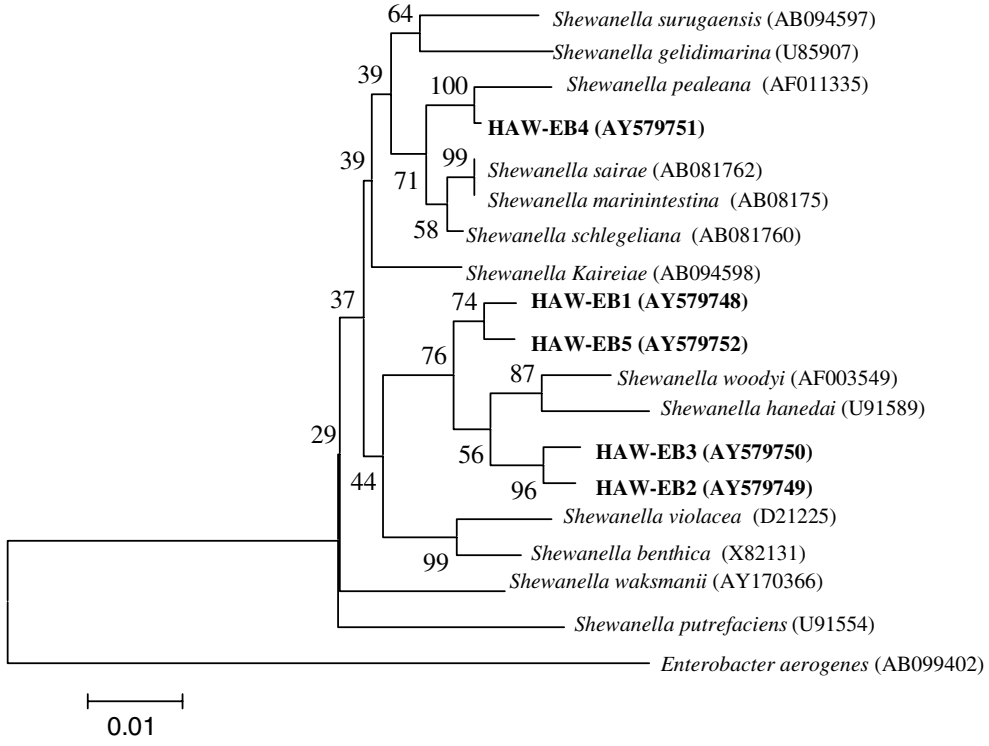

Fig. 2. Phylogeny of aerobic isolates. The phylogenetic tree was generated based on pair wise nucleotide distance of Kimura 2-parameter using the neighbor-joining method included in the MEGA2 software package. The bar indicates a difference of 1 nucleotide per 100. The number beside the node is the statistical bootstrap value. In brackets are the GeneBank accession numbers of the16S rRNA genes.

diameter of 0.5 μm) and yellow bacterium. It appeared to be a slow-growing bacterium at temperatures ranging from 4 to 21 °C in the present media.

Many other bacteria (Group III, HAW-EB6-13, $RDX^{-}HMX^{-}$) were also isolated from the initially detected aerobic and anaerobic colonies. They were oxidase-negative, catalase-positive, colorless or whitish in color, rod-shaped (1.0–6.0 μm long with diameters ranging from 0.5 to 2.5 μm) and Gram-negative bacteria, capable of both aerobic and anaerobic growth at low temperatures (4 and 10 °C). Although some contained hydrogenase activity (they produced H_2 anaerobically when grown in RDX degradation medium containing peptone and yeast extract as carbon and nitrogen sources) similar to previously reported RDX-degrading anaerobes [18], they seemed to transform RDX and HMX poorly.

3.2. Phylogeny of cyclic nitramine-degrading isolates

The five RDX-degrading isolates within group I were further analyzed for their phylogenetic affiliation by comparing their 16S rRNA genes (1.2–1.3 kb long) (Fig. 2) with those of other related bacteria. Phylogenetic analyses indicated that the five isolates from group I fell within the genus *Shewanella* of the gamma Proteobacteria (Fig. 2). Isolate HAW-EB4 was clustered together with *S. pealeana*, *S. sairae*, *S. marinintestina*, and *S. schlegeliana* with high similarities (99%). Isolates HAW-EB2 and HAW-EB3 were clustered together with *S. hanedai* and *S. woodyi* (97.2–97.6% similar). Isolates HAW-EB1 and HAW-EB5 clustered together, matching *S. woodyi*, *S. hanedai*, *S. kaireiae*, *S. schlegeliana*, and *S. violacea* with similarity from 96.3% to 97.4%. *Shewanella* is known for its dissimilatory reduction of manganese (IV) and iron (III) oxide [31–33]. Iron was found as a major metal (40 $g\,kg^{-1}$ (dry weight)) in marine sediment used in this study and thus might favor growth of iron (III)-reducing *Shewanella* in the sediment [33]. Since RDX is a potent electron acceptor, it might serve as a substrate to the iron (III)-reducing *Shewanella*, thus creating a mechanism for in situ degradation of RDX in marine sediment.

Phylogenetic analysis of three of the four obligatory anaerobic, RDX and HMX degrading isolates of group II is shown in Fig. 3. Isolate HAW-EB18 grouped within the Desulfovibrionale cluster of the delta Proteobacteria and was closely related to the sulfate-reducing bacteria

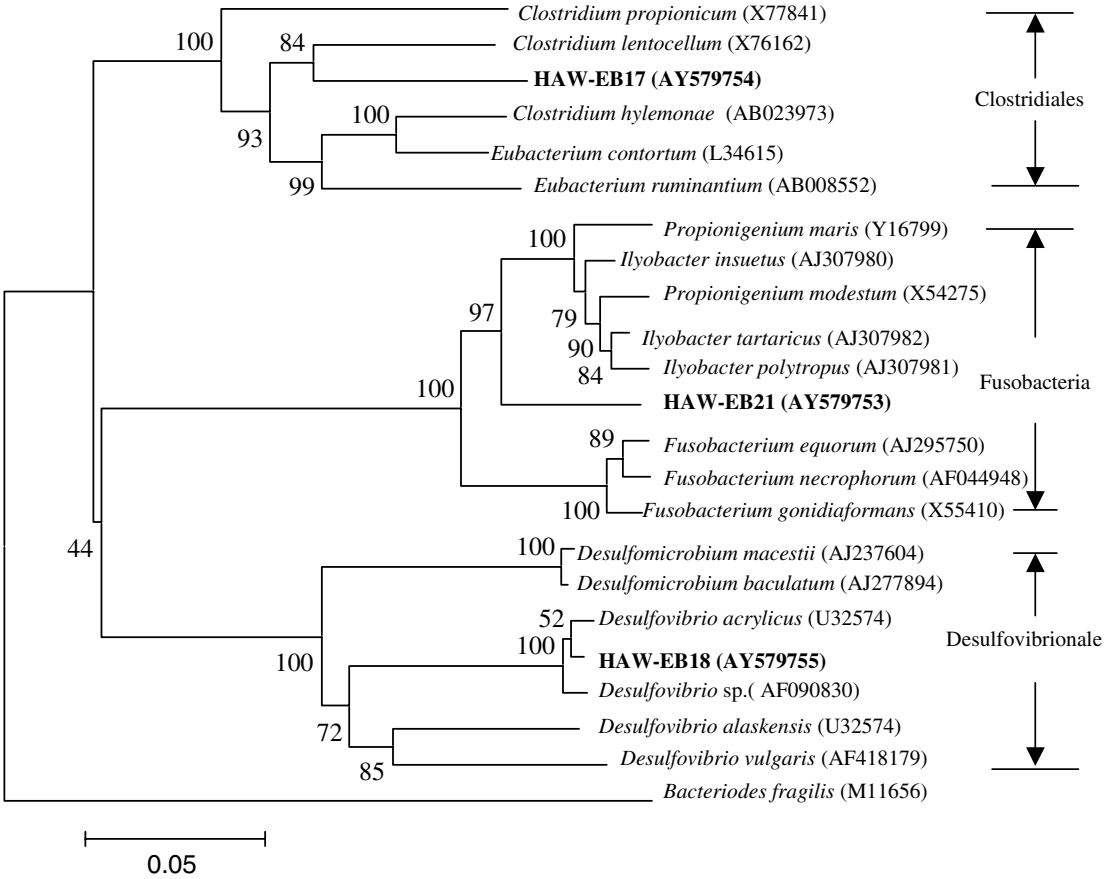

Fig. 3. Phylogeny of obligately anaerobic isolates. The phylogenetic tree was generated using the same methods described in Fig. 2. The bar indicates the difference of 5 nucleotides per 100. In brackets are the GeneBank accession numbers of the16S rRNA genes.

Desulfovibrio acrylicus (99% similar) and *Desulfovibrio* sp. TBP-1 (98% similar). Sulfate reduction is an important microbial process in marine sediment [7,37,43]. Detection of the sulfate-reducing bacterium (HAW-EB18) as a cyclic nitramine degrader indicates the involvement of the sulfate-reducing microbial process in the in situ natural attenuation of RDX and HMX in marine sediment.

The obligatory anaerobic and H_2-producing fermentative isolate HAW-EB21 grouped within the cluster of Fusobacteria with species of *Propionigenium* and *Ilyobacter* as close matches (92.4–92.9% similar): *P. maris*, *P. modestum*, *I. insuetus*, *I. polytropus*, *I. tartaricus*. This demonstrates that strictly anaerobic H_2-producing bacteria also contribute to the natural attenuation of cyclic nitramine explosives. Previously we observed that an H_2-producing obligate anaerobe *Clostridium* in anaerobic sludge degraded RDX [18].

The 16S rRNA gene of the obligate anaerobic isolate, HAW-EB17, clustered together with bacteria belonging to the order Clostridiales with species of *Clostridium* (*C. lentocellum*, *C. hylemonae*, *C. propionicum*) and *Eubacterium* (*E. contortum*, *E. ruminantium*) as close matches (89.5% similar). The result suggests that bacteria of the order Clostridiales are also involved in the natural attenuation of cyclic nitramine explosives, consistent with

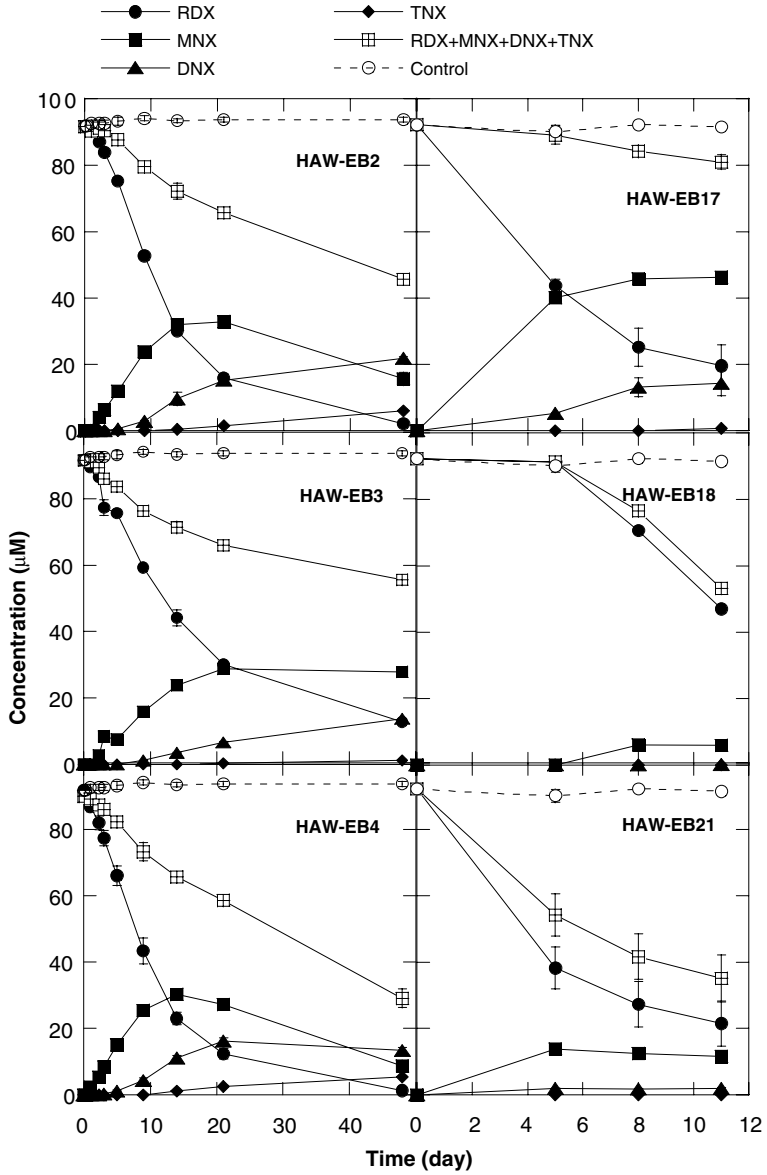

Fig. 4. Biotransformation of RDX by psychrophilic isolates growing in marine medium 2216 at 10 °C. Isolates HAW-EB2, HAW-EB3 and HAW-EB4 were grown under oxygen limited conditions. Isolates HAW-EB17, EB18 and EB21 were incubated under strictly anaerobic conditions. The lower amounts of total RDX + MNX + DNX + TNX from the total amount of RDX in the control were due to the formation of CO_2 (see Fig. 5), MEDINA and NDAB (see Fig. 6).

our previous observation in anaerobic sludge that *Clostridium* spp. were RDX degraders [18].

3.3. Cyclic nitramine biodegradation by isolates at low temperature

Isolates HAW-EB2, HAW-EB3 and HAW-EB4 grew and removed RDX (Fig. 4), but not HMX (data not shown), under oxygen-limited conditions whereas isolates HAW-EB17, HAW-EB18 and HAW-EB21 grew and removed both RDX (Fig. 4) and HMX (data not shown) under strictly anaerobic conditions. Removal of RDX by isolates of group I and II was accompanied by the formation of nitroso derivatives (MNX, DNX and TNX, Fig. 1) with MNX as the major product (Fig. 4). Nitroso products were not persistent, and they disappeared from the growing cultures of all isolates after a longer incubation (data not shown). The two tested obligatory anaerobic isolates HAW-EB18 and HAW-EB21 slowly transformed HMX to produce the mononitroso derivative (NO-HMX, Fig. 1, data not shown). In addition to the nitroso products, RDX removal by growing cells of isolates of groups I and II was accompanied by mineralization (3.7–45.2%, 82 days) determined as liberated $^{14}CO_2$, with the highest amount observed with isolate HAW-EB4 (45.2%, 82 days) (Fig. 5).

During RDX transformation with resting cells of group I isolates, we detected the ring cleavage products MEDINA, NDAB, N_2O, and a trace amount of nitrite in addition to the nitroso products (Figs. 1 and 6). Of the RDX transformed by isolate HAW-EB2, most (44–45%) was found as nitroso products (MNX, DNX and TNX) with a small part (19–25%) as ring cleavage products (MEDINA and NDAB). However, transformation of RDX with isolates HAW-EB4 and HAW-EB5 mainly produced the cleavage products MEDINA and NDAB (43–53%) and only a small percentage of nitroso products (12–27%). Total amounts of MEDINA, NDAB and the nitroso products accounted for up to 80% (molar) of RDX transformed, whereas N_2O accounted for 18–26% of total N of RDX removed. As we reported previously, N_2O originated from the decomposition of MEDINA [25,34].

Resting cells of isolates HAW-EB2, HAW-EB4 and HAW-EB5 removed nitrite at rates (33, 7.8, and 12 $\mu M\,h^{-1}$, respectively) 20–70 times faster than those

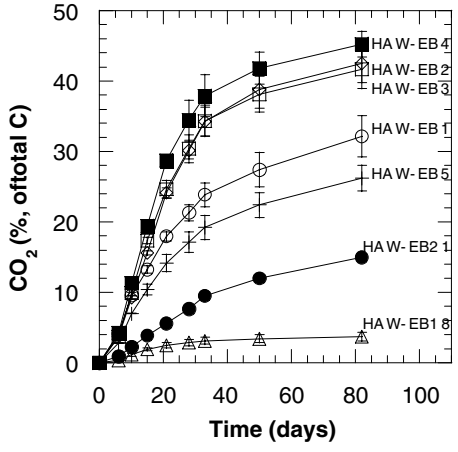

Fig. 5. RDX mineralization by growing psychrophilic isolates (the conditions were the same as those of Fig. 4).

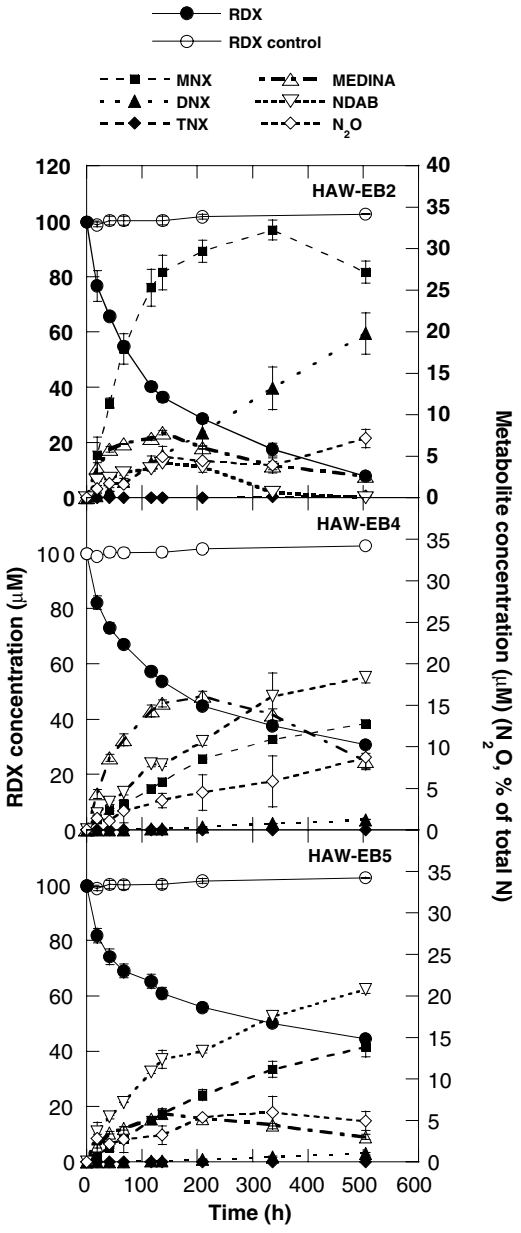

Fig. 6. Formation of MEDINA and NDAB during static and anaerobic incubation of RDX at 10 °C with washed cells of isolates HAW-EB2, HAW-EB4 and HAW-EB5 suspended in marine salts medium.

(0.43–0.47 $\mu M\,h^{-1}$) of RDX removal under the same conditions, explaining why only traces of nitrite were detected in this study. We previously reported that denitration of either RDX or its mononitroso product (MNX) led to ring cleavage and the production of MEDINA and NDAB [35,36]. We suggest that similar denitration reactions occurred with RDX and its mononitroso product MNX prior to ring cleavage by the psychrophilic isolates.

As the above discussion reveals, isolates of *Shewanella* and Fusobacteria obtained in the present study metabolized RDX with substantial mineralization. In contrast, other reported bacteria such as *Clostridium* or some members of the *Enterobacteriaceae* family reduced RDX mainly to nitroso products [17,20,21]. The present RDX-mineralizing isolates are also different from another RDX-mineralizing isolate, *K. pneumoniae* strain SCZ-1 in that the latter seemed to act poorly in reducing RDX to nitroso products [11]. Formation of both MEDINA and NDAB in RDX biodegradation by a single culture has not been reported previously.

Shewanella, as present marine sediment isolate HAW-EB1-5, is known to exist in other non-contaminated marine water and sediment and for its ability to degrade pollutants such as halogenated and petroleum pollutants [31,32,38–41]. Species of *Clostridium and Desulfovibrio* similar to the present anaerobic isolate HAW-EB17 and EB-18, were previously found in domestic anaerobic sludge [17–19]. Soil bacteria at non-contaminated sites were reported to be capable of degrading RDX under anaerobic conditions [42]. These findings together suggest that microorganisms capable of degrading cyclic nitramine explosives under anaerobic conditions are widely present in the environment including the present marine sediment, thus the cyclic nitramine explosives if released from UXO to the sediment would be degraded by indigenous anaerobic bacteria, such as *Shewanella* and sulfate reducers.

Our present results clearly demonstrated that psychrophilic bacteria capable of degrading cyclic nitramines were present in the marine sediment, and their presence might have contributed to the natural attenuation of these chemicals. This may explain the absence of RDX and HMX in the collected Halifax marine sediment despite the known practice of dumping ammunition and UXO at the site. Abiotic reactions and adsorption might also affect the detection of the two chemicals in the sediment, but they seem to be less significant in comparison to biodegradation. For instance, adsorption of the two cyclic nitramines RDX and HMX on soil (no data is yet available on sediment) has thus far been found to be reversible and low (sorption distribution coefficients $<2.5\,l\,kg^{-1}$) [24,30]. Also the half-life of RDX (0.59 mg in 10 g wet sediment) in sterilized sediment was 9 times that in non-sterilized sediment [22].

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